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During the third year of the funded period, we focused on the characterization of interactions between hRAD51 and the five hRAD51 paralogs. We detected strong interactions suggesting a stable complex, and weaker interactions. Some of these weaker interaction signals between hRAD51 paralogs increased in the presence of ATP and decreased in the presence of ADP which may indicate a regulatory role for adenosine nucleotides. We also continued in our effort to purify the paralogs and heterodimers thereof. Examination of the functional significance of these interactions and the role of adenosine nucleotides is currently in progress.

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(4) INTRODUCTION:

DNA repair is essential for genomic integrity, and failure of repair pathways may lead to a mutator phenotype and to tumorigenesis (1, 2). Homologous DNA recombination (HR) is a prominent pathway for the repair of double-strand break (DSB) and other DNA lesions and is dependent on human RAD52 epistasis group proteins including hRAD51 and its five paralogs (3-6). Evidence for the involvement of both BRCA1 and BRCA2 in hRAD51-mediated repair processes is accumulating (7-9). The purpose of this study is to characterize physical and functional interactions among hRAD51 paralogs and BRCA1/BRCA2 in order to better understand HR.

(5) BODY

Brief summary of previously reported work

Aim I: done, results have been reported in Cancer Res. (Ref.11). The coding region of the hRAD51 gene has been examined for mutations in tumor tissues with high frequencies of 15q15 deletions, and the promoter region has been tested for hypermethylation. No changes have been found in the tumors compared to normal tissues.

Aim II: partially done, results have been reported. All known human RAD51 homologs involved in mitotic recombination have been cloned into appropriate vectors and overexpressed in bacteria or in a baculovirus system. Polyclonal antibodies against these proteins have been generated and characterized. Monoclonal antibodies have become available. Previously unknown chromosomal locations of RAD51 homologs have been determined. Purification of XRCC2 has been reported.

Aim III: partially done. Basic interactions between hRAD51 and its human paralogs have been reported. We have cloned members of the human RAD52 epistasis group into appropriate expression vectors: hRAD51, hRAD51B (a.k.a. hRAD51L1 or hRAD51-H2), hRAD51C (a.k.a. hRAD51L2), hRAD51D (a.k.a. hRAD51-H3, hRAD51L3), hXRCC2, hXRCC3 as well as hRAD52 and hRAD54. This collection allowed us to clearly identify static interactions between these six human mitotic RecA homologs, hRAD52, hRAD54 (Aim IIIa). Interaction studies were done using an *in vitro* GST-fusion-IVTT method as described (10, 11). In this assay, hRAD51 gave a strong signal with itself as expected. We also found (weaker) interactions with hRAD51B, hRAD51D, hXRCC3, and hRAD52. hRAD51B showed strong interactions with hRAD51C and hRAD51D and weaker signals with hXRCC3. hRAD51C interacted strongly with hRAD51B and hRAD51D and less strongly with XRCC3. hRAD51D interactions were detected with all other hRAD51 homologs. The strongest signal was seen with hXRCC2, and hXRCC2 seems to interact only with hRAD51D. hXRCC3 also seems to be able to interact with all hRAD51 homologs, but seems to bind best to hRAD51D. In addition, we detected signals in the hRAD52 and hRAD54 lanes. hRAD52 bound to hRAD51, hRAD51B, hRAD51D, hXRCC3 and itself.

Summary of current work

Aim IIIa: Interaction studies. Since all RAD51 homologs have highly conserved ATP binding domains (Walker boxes) and ATP binding/hydrolysis seems to have a crucial role during homologous recombination (12), we further investigated whether interactions between the hRAD51 homologs could be modified by adenosine-nucleotide. We found that GST-hRAD51 precipitates IVTT-hRAD51D in the presence of ADP plus sodium aluminum tetrafluoride ($NaAlF_4$; Figure 1, Lanes 4 and 5). The function of $NaAlF_4$ is unknown. However, it is generally regarded that $NaAlF_4$ stabilizes NDP-bound Walker A/B motif proteins in a pseudo-transition state.

hRAD51-GST + hRAD51D (IVTT)

$NaAlF_4$				+	+	
ADP				+		+
ATPγS			+			
ATP		+			+	

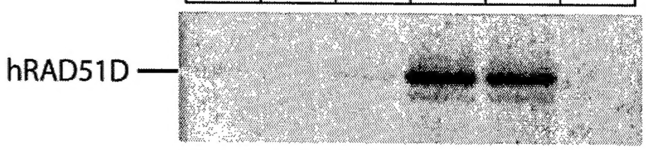

Figure 1. GST/IVTT precipitation analysis. A GST fusion construct of hRAD51 (GST-hRAD51) was bound to glutathione beads and exposed to ^{35}S-labeled *in vitro* transcribed translated hRAD51D (IVTT-hRAD51D) in the presence of 1mM ADP, ATP, ATPγS and/or ADP-$NaAlF_4$ as indicated. Precipitated proteins were separated by PAGE and visualized using a PhosphoImager system. No significant IVTT material was precipitated by GST alone (data not shown).

As mentioned above, hRAD51D forms a very stable heterodimer with hXRCC2. We have purified the hRAD51D/hXRCC2 heterodimer using a baculovirus expression system. Immuno-precipitation experiments (Figure 2) support the conclusion that hRAD51D/hXRCC2 interacts with the hRAD51-ADP- $NaAlF_4$ transition state.

	purified proteins			IP with hRAD51 antibody						
Mg-ATP							+			
Mg-ADP, $NaAlF_4$								+		+
hRAD51	+			+	+	+	+	+	+	+
hXRCC2		+			+		+	+		
hXRCC2/hRAD51D			+			+			+	+

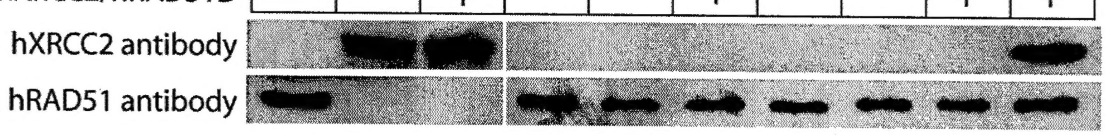

Figure 2. Immunoprecipitation of hXRCC2 and hXRCC2-hRAD51D with hRAD51. Purified hRAD51 was bound to Protein A beads preincubated with hRAD51 antibody and subsequently exposed to purified hXRCC2 or hXRCC2-hRAD51D in the absence of adenosine nucleotide or in the presence of 1mM ADP or ADP-$NaAlF_4$ as indicated. Precipitated proteins were separated on SDS-PAGE and probed with a monoclonal antibody to hXRCC2 (hXRCC2; upper panel) or the hRAD51 antibody (hRAD51; lower panel) Lanes 1-3 contain purified proteins which had not been subjected to immunoprecipitation.

Preliminary data have also shown that binding of hRAD51 to the hXRCC3-GST fusion protein is influenced by adenosine nucleotides. Binding of hRAD51 to hXRCC3 is increased in the presence of ADP and ATP in the presence of $NaAlF_4$ (Figure 8).

These data indicate a regulatory role of ATP hydrolysis and/or binding. We currently perform experiments using monoclonal antibodies to XRCC3 and hRAD51C, respectively, to further confirm these findings.

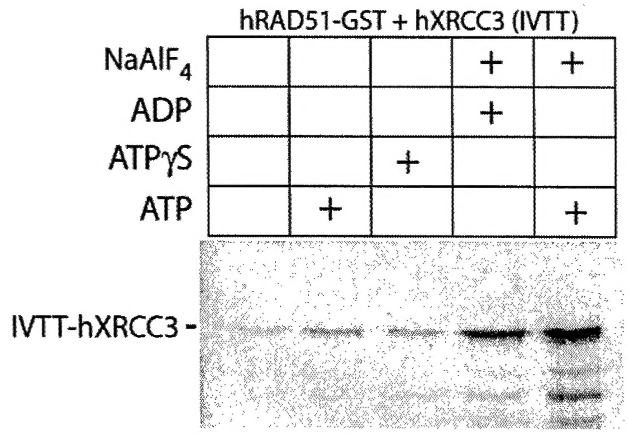

Figure 3. GST/IVTT precipitation analysis (see legend to Figure 1).

In addition, co-immunoprecipitation experiments using specific antibodies and HeLa cell extracts are currently in progress.

Aim III b) Purification and characterization of hRAD51 derivatives. Purification of hXRCC2, hRAD51D/hXRCC2 heterodimer, hRAD51C/hXRCC3 heterodimer have been completed. Purification of hRAD51B/hRAD51C heterodimer and biochemical studies to characterize the human RAD51 paralogs are currently in progress.

Aim IV a) Interactions of hRAD51 derivatives with BRCA2, BARD1, and RPA. In order to test BRCA2 in our GST interaction assay, it was necessary to divide the 10.3kb BRCA2 ORF into four overlapping fragments of approximately 3kb each since we could not generate a full length labeled BRCA2 IVTT protein in sufficient quantities. Interaction experiments with these fragments, the hRAD51 paralogs, BARD1, and RPA are currently in progress.

(6) KEY RESEARCH ACCOMPLISHMENTS:

- Interactions between hRAD51 and its homologs have been further characterized (Aim IIIa).

- Interactions between hRAD51 homologs seem to be modified in the presence of ATP or ADP (Figures 1-3). These findings support a regulatory role for adenosine nucleotides during HR.

(7) REPORTABLE OUTCOMES

All findings listed under (6) are reportable. Data describing interactions of hRAD51 and paralogs have been presented at the Era of Hope meeting in Orlando (Sept.25-28, 2002). A manuscript about the function of hXRCC2 (not described here) has been submitted to Molecular Cell for publication.

(8) CONCLUSIONS

Multiple interactions exist between hRAD51 and the human hRAD51 paralogs which seem to be in part regulated by adenosine nucleotides. Purification and biochemical characterization of these proteins is in progress in order to study the functional relevance of these interactions in the process of homologous recombination.

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(10) APPENDICES

none.